Lasers in Surgery and Medicine 26:83–89 (2000)

Localization of Experimental Submucosal Esophageal Tumor in Rabbits by Using Mono-L-aspartyl Chlorin e6 and Long-Wavelength Photodynamic Excitation

Ilyar Sheyhedin , MD,[1,2] Tetsuya Okunaka, MD,[2]* Harubumi Kato, MD,[2] Yutaka Yamamoto, MD,[2] Nobuyuki Sakaniwa, MD,[2] Chimori Konaka, MD,[2] and Katsuo Aizawa, PhD[3]

[1]Department of Thoracic Surgery, Xinjiang Medical University, Urumqi 830000, China
[2]Department of Surgery, Tokyo Medical University, Tokyo 160-0023, Japan
[3]Department of Physiology, Tokyo Medical University, Tokyo 160-0022, Japan

Background and Objective: **To increase the applicability of photodynamic diagnosis with regard to deep-seated tumor, we illuminated tumors with a long-wavelength laser beam after photosensitization with mono-L-aspartyl chlorin e6 (NPe6).**
Study Design/Materials and Methods: **Rabbits with VX2 esophageal tumors were divided into four groups. The control group was not treated, and the other three groups were injected with 1, 2.5, and 5 mg/kg mono-L-aspartyl chlorin e6 (NPe6), respectively. After excitation with a 664-nm laser beam (10 mW, 10 seconds), the fluorescence image and the relative fluorescence intensity (tumor/normal tissue) were recorded every 2 hours up to 8 hours by a newly developed diode laser endoscopic fluorescence imaging system. The tissue concentration of NPe6 was examined by high performance liquid chromatography at 2, 4, and 6 hours after injection with 1 and 5 mg/kg NPe6.**
Results: **The diode laser endoscopic fluorescence imaging system was able to selectively detect fluorescence from submucosal tumor by comparison with the surrounding normal mucosa after NPe6 injection. The fluorescence intensity correlated with NPe6 dose, selectively accumulated in the tumor tissue and relative intensity peaked at 6 hours after injection. No fluorescent images were detected in controls.**
Conclusion: **Given intravenously, NPe6 at a dose of 5 mg/kg and excited with a 664-nm wavelength laser beam 6 hours later can define experimentally induced deep-seated esophageal carcinoma in rabbits, by using an endoscopic fluorescence imaging system. Lasers Surg. Med. 26: 83–89, 2000.** **© 2000 Wiley-Liss, Inc.**

Key words: fluorescence imaging; NPe6; photodynamic diagnosis (PDD); photosensitizer

INTRODUCTION

Photodynamic therapy (PDT) uses a specific wavelength laser beam to excite certain photosensitizers [1], and its effects have been confirmed [2,3]. However, these studies mainly emphasized the therapeutic effects, largely neglecting photodynamic diagnosis (PDD) potential. PDD was generally devoted to in vivo sensitizer pharmacokinetic aspects [4,5], photobleaching follow-up [5,6], or spectroscopy for tissue diagnosis [7,8].

Laser beams with a wavelength close to 400 nm are normally uses in PDD [9–14]. Because these wavelengths can only penetrate up to some hundreds of micrometers, the clinical applications

*Correspondence to: Tetsuya Okunaka, Department of Surgery, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
E-mail: okunaka@tokyo-med.ac.jp

Accepted 5 October 1999

are confined to the detection of surface lesions [7]. Moreover, the absorption bands of hemoglobin and many endogenous fluorescent substances are also around 400 nm and, thus, further block the excitation light beam [15,16] from penetration and limit the ability to detect more deeply located tumors.

New photosensitizers with light absorption at wavelengths close to 700 nm allow deep light penetration [4,17–20]. One of these sensitizers, mono-L-aspartyl chlorin e6 (NPe6) is an effective agent with a Q band at 664 nm. NPe6-PDT has been shown to be effective both in vivo and in vitro [21–24]. However, the applications of NPe6-PDD are limited, and when we excited tumor tissue with 405 nm light in lung cancer patients, the tumor margin was unclear [25]. To clarify the usefulness of fluorescent localization of deep-seated submucosal esophageal carcinoma in rabbits, we assessed the tumor-localizing ability of NPe6 with a new diode laser endoscopic fluorescence imaging system.

MATERIALS AND METHODS

Photosensitizer

NPe6 was provided by Meiji Seika Kaisha, Ltd., Tokyo, Japan. It is a dark blue-green purified compound with a molecular weight of 799.70. The drug was dissolved in normal saline immediately before use to avoid the possibility of light-induced degradation.

Animal Model of Esophageal Tumor

Japanese white male rabbits, each weighing 2.5–3.0 kg, were used. VX2 tumor cells (Funabashi Farm Co., Ltd., Chiba, Japan) originating from squamous cell carcinoma were obtained as an in vivo transfer [26,27]. After laparotomy under Nembutal anesthesia, a tumor cell suspension consisting of 5×10^5 VX2 cells/0.1 ml was injected from the esophageal tunica adventitia into the submucosa of the lower thoracic esophagus of rabbits. A week later, the implanted tumors were 3–5 mm in lateral dimension and 3–4 mm in thickness.

Diode Laser Endoscopic Fluorescence Imaging System

The diode laser endoscopic fluorescence imaging system consists of a light source system, a fluorescence imaging system (Matsushita Industrial Equipment Co., Ltd., Osaka, Japan) and an

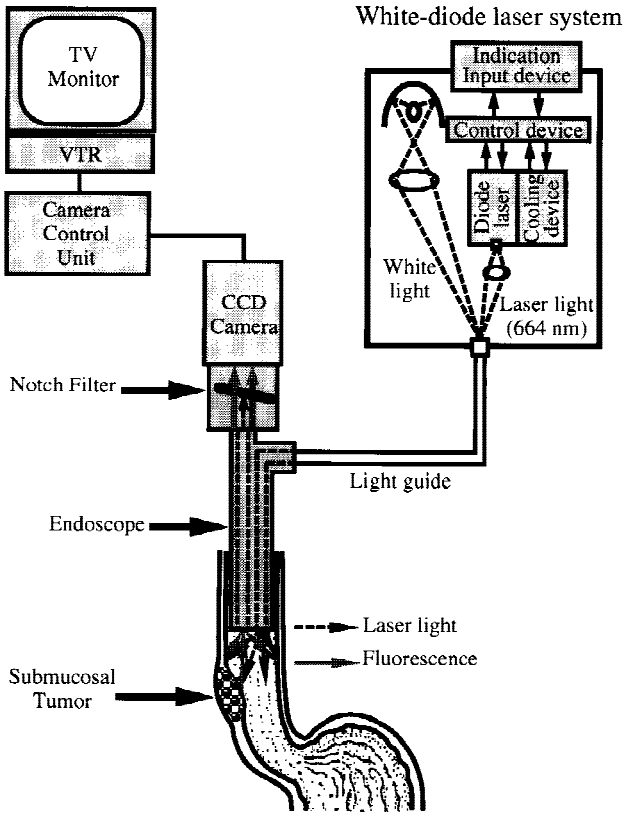

Fig. 1. Diagram of the diode laser endoscopic fluorescence imaging system. CCD, charge-coupled device; VTR, videotape recorder.

Olympus BF 1T10 fiberoptic bronchoscope 6 mm in diameter (Olympus Optical Co, Tokyo, Japan) (Fig. 1). This system does not induce autofluorescence and has excellent in vivo transmittance because of long-wavelength excitation at 664 nm. The light source system contains two different light sources, one of which emits white light from a halogen lamp (100 V, 150 W), whereas the other is a tunable laser diode light source (664 nm, 140 mW) that can emit a wavelength from 660 nm to 675 nm. These light beams are delivered to the entire view area, including the lesion, by means of the light guide of the bronchoscope, and fluorescence from the excited lesions are detected by the fluorescence imaging system. The system contains a notch filter (Kaiser Optical Systems, Inc., USA), which is placed between the bronchoscope and the charge-coupled device (CCD) camera (Panasonic WV-BP 100, Matsushita Industrial Equipment). The filter can selectively cut off the excitation light at 664 nm. The fluorescence image is captured through the CCD camera connected to a TV monitor. The CCD images were saved on videotape and later digitized and ana-

lyzed by a computer. In this study, the diode laser wavelength was adjusted to 664 nm to suit the absorption band of NPe6.

Measurements of Fluorescence Intensity of NPe6 and Histology of the Lesions

Twelve tumor-bearing rabbits were divided into four groups (three in each group). Three groups were given NPe6 intravenously at a dose of 1, 2.5, and 5 mg/kg, respectively. Esophageal fluorescence images were observed every 2 hours from 2 to 8 hours after Nembutal anesthesia. One group of rabbits received no drug and served as control animals. The images were recorded on a videotape and transformed into digital images by using MediaGrabber software (Apple Computer, Inc., Cupertino, CA). We calculated the mean of tumor fluorescence intensity of NPe6 by measuring two identical circles (diameter = 2 mm), where one circle was in the brightest fluorescence part of the tumor while the other was in on area with slightly weaker fluorescence intensity. The mean of two identical circular adjacent areas without visible fluorescence, located 2 mm apart from the visible fluorescence, was chosen as normal. Data were analyzed with NIH Image 1.57 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). After measurements, all rabbits were killed by an overdose of Nembutal sodium. The esophagus, including lesions and adjacent normal tissue, was removed and fixed in buffered formalin, sectioned at 3 μm thickness, then stained with hematoxylin and eosin for histologic examination.

Measurements of NPe6 Concentration in Tissues

Twenty-three tumor-bearing rabbits were divided into control (n = 5) and tissue concentration experimental groups (n = 18). The latter group was further divided into group A (1 mg/kg NPe6, intravenously) and group B (5 mg/kg i.v.). Tissue NPe6 concentrations of tumor and normal esophagus were checked at 2, 4, and 6 hours after injection by high-performance liquid chromatography (HPLC) analysis. The specimens were stored at ×80°C until use. Tissue (0.1 g) was minced and homogenized in 0.1 ml of 0.4% ethylenediaminetetraacetic acid/50 mM HEPES (pH 7.4) and then extracted with 5.0 ml of a chloroform/methanol mixture (1:1, v/v). Then 20 ml of distilled water was added to the homogenate by vortex mixture for 5 seconds. After centrifugation at 3,000 rpm for 15 minutes, the supernatant was removed. The precipitate was extracted again by the same method. The first and second supernates were filtered through a SEP-PAK Cartridge tC18 (Waters Co., Milford, MA) for adsorption of NPe6. The adsorbed NPe6 was extracted with 2 ml of methanol. These extracted samples were injected into an HPLC system (Hitachi, Ltd., Tokyo, Japan).

Statistical Analysis

One-way analysis of variance (ANOVA) was used for statistical analysis (Fisher for Windows, version 1.2). A P value of less than 0.002 was considered significant.

RESULTS

Figure 2A,B showed the endoscopic white light and fluorescent images of the esophageal lumen and a submucosal tumor. The endoscopic fluorescence imaging system distinguished the fluorescent images of submucosal tumors and surrounding normal mucosa after NPe6 injection. No fluorescence was detected in the esophagus of control rabbits not receiving injections of NPe6. The 3 × 2 mm tumor was located about 1.2 mm below the esophageal mucosa. The mucosa surface was intact (Fig. 2C).

The relative fluorescence intensity ratios of esophageal submucosal tumor and surrounding normal mucosa (T/N) increased after injection of each dose of NPe6. Peak values were reached at 6 hours, and thereafter decreased (Fig. 3). Peak values of relative fluorescence intensity ratios at 6 hours after injection of 1 and 5 mg/kg NPe6 showed significant differences (1.76 vs. 3.51, P = 0.0014, one-way ANOVA). There was no significant difference between the 2.5 mg/kg and 1 mg/kg NPe6 dose. The tissue concentrations of NPe6 in esophageal tumor and surrounding normal tissue after injection of 1 and 5 mg/kg NPe6 decreased from 2 to 6 hours, but the decline in tumor was slower than in normal tissue (Fig. 4A). Therefore, the T/N ratios of tissue concentrations of NPe6 in esophageal tumor and surrounding normal tissue increased from 2 to 6 hours (Fig. 4B). There were significant differences of the tissue NPe6 concentration ratios (T/N) between these 2 doses at 6 hours after injection (P = 0.0016). Both the T/N ratios of NPe6 fluorescence intensity and tissue concentration reached their peak value with the highest dose (5 mg/kg NPe6) 6 hours after injection and revealed good correlation.

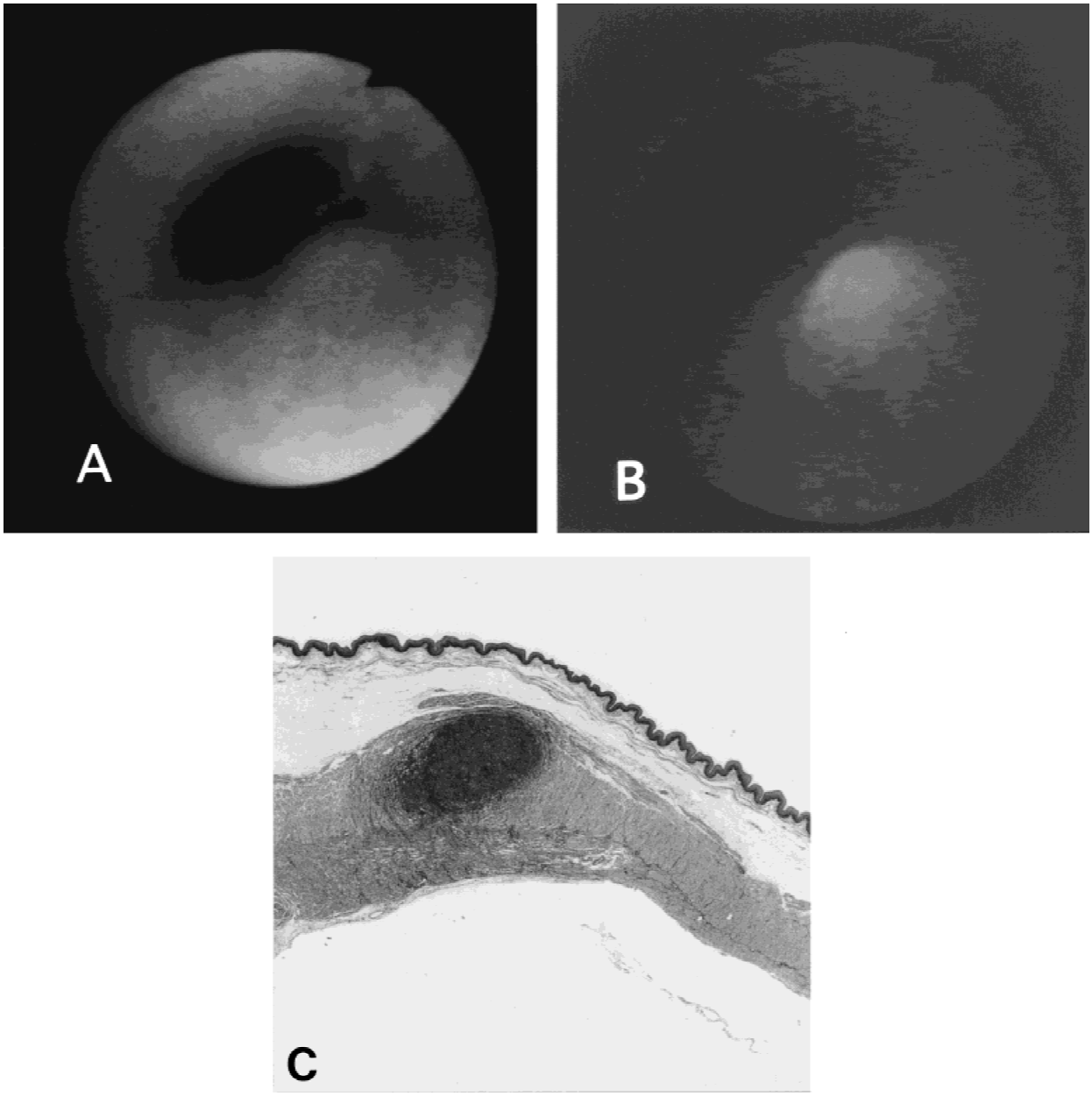

Fig. 2. Endoscopic photographs showed the white light image (**A**) and fluorescence image (**B**) of the esophagus of rabbits at 6 hours after administration of 5 mg/kg NPe6 on excitation with a 664-nm laser beam. The submucosal tumor was 3 × 2 mm in size. The mucosa is intact. **C:** The tumor was located about 1.2 mm below the esophageal mucosa.

DISCUSSION

PDD is a low-invasive technique that can be used to distinguish normal from malignant tissue. This technique uses the principle that when certain compounds are excited by light, they exhibit a characteristic fluorescence emission [28,29]. Autofluorescence from normal tissues and hemoglobin has an absorption peak near 400 nm, thus, PDD that uses this wavelength can be used to detect only superficial changes [15,25]. The present diode laser endoscopic fluorescence imaging system overcomes this problems because of long wavelength excitation of 664 nm. The power from the tip of the bronchoscope in white light illumination is 1 mW, whereas in laser light it is 20 mW or less in case of the excited fluorescence observation. This power is low and will not cause tissue damage. In other words, not only is the photoradiation intensity very low but also the total pho-

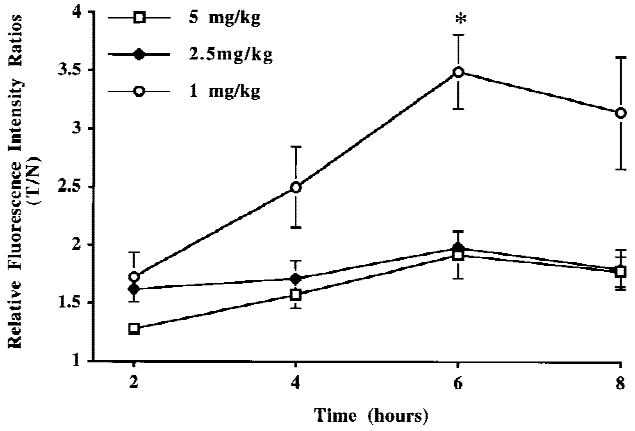

Fig. 3. The fluorescence intensity ratios of mono-L-aspartyl chlorin e6 (NPe6) in esophageal submucosal tumors and surrounding normal tissues (T/N) after NPe6 administration. Results are expressed as means ± SD. The asterisk indicates $P < 0.002$.

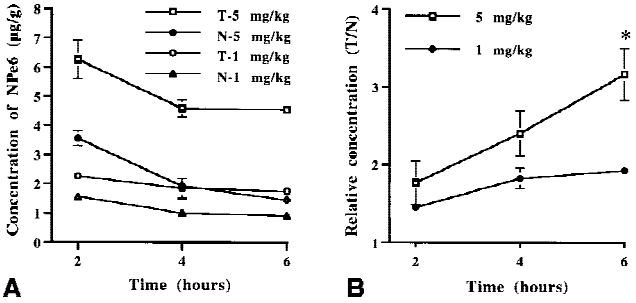

Fig. 4. **A,B:** The tissue concentrations of mono-L-aspartyl chlorin e6 (NPe6) in esophageal submucosal tumors and surrounding normal tissues after NPe6 administration measured by high performance liquid chromatography. Results are expressed as means ± SD. The asterisk indicates $P < 0.002$.

toradiation energy, including photoradiation time, is less than 1/100 that of PDT; thus, potential effects on tissue are limited.

NPe6 is an effective photosensitizer that possesses properties such as chemical purity and a major absorption band at 664 nm. These features are potentially exploitable for PDT [17,23]. In comparison to hematoporphyrin derivative (HpD), NPe6 does not cause prolonged normal skin photosensitization [21,30]. The single narrow peak and high intensity of the NPe6 emission spectrum make it easily recognizable, in comparison to the emission spectrum of HpD, which has a biphasic pattern with two emission peaks at 630 nm and 960 nm. The biphasic emitted fluorescence of HpD, with the main peak at 630 nm, is weak [31]. Furthermore, the peak wavelength of the fluorescence emission spectrum of NPe6 in tissue is 675 nm [8], 45 nm longer than that of HpD, which allows better emitted fluorescence detection because of its better tissue penetration.

The dose of photosensitizer and the distribution in tumor and normal tissue (T/N ratio) after injection may determine the effect of PDD and even PDT for malignant tumors. Our results showed that the relative fluorescence intensity of esophageal submucosal tumors and surrounding normal mucosa gradually increased from 2 to 6 hours after injection of different doses of NPe6. HPLC analysis showed that the concentration of NPe6 in tumor was significantly higher than in the surrounding normal esophagus. Although the concentrations of NPe6 in tumor and normal tissue decreased gradually after injection, the T/N ratios of NPe6 concentration increased. The maximal T/N ratio value of tissue NPe6 concentration was obtained with the highest dose (5 mg/kg NPe6) 6 hours after injection. This result was similar to the data reported by Gomer and Ferrario [21], who used a mouse tumor model. In our study, the absolute concentration of NPe6 in tissue was higher than in the report of Gomer and Ferrario, probably because of the different animal model we used.

Our study is the first report on long-wavelength excitation PDD for experimental esophageal carcinoma in vivo by using NPe6 as the tumor-localizing photosensitizer. We found that implantation of tumor cells through the tunica adventitia side of the rabbit esophagus after laparotomy has the following benefits: (1) the method is technically easy, (2) the integrity of the mucosa is preserved, (3) the tumors develop within 1 week.

Our system was developed with clinical applications in mind and was convenient to use. We used a 664-nm laser beam excitation wavelength for NPe6-PDT. Therefore, we can perform diagnosis and treatment simultaneously. Furthermore, the notch filter in our system can let visible light and fluorescence pass through, cutting off only the 664-nm excitation light beam. Alternate observation with visible light and fluorescence can be easily done without changing instruments.

In summary, it is possible to diagnose relatively deep-seated esophageal tumors by diode laser endoscopic fluorescence imaging system through excitation of NPe6 by 664-nm wavelength light. Our data showed that optimal differentiation between tumor and normal tissues was

achieved at 6 hours after injection of 5 mg/kg NPe6.

ACKNOWLEDGMENTS

The authors are indebted to Professor J.P. Barron of Tokyo Medical University and Dr. Takwah Wong of the National Cheng-Kung University, Taiwan, for their review of the manuscript. The authors also thank Matsushita Industrial Equipment Co., Ltd., Osaka, Japan and Meiji Seika Kaisha, Ltd., Tokyo, Japan for supplies of equipment and drugs.

REFERENCES

1. Dougherty TJ. Photosensitizers: therapy and detection of malignant tumors. Photochem Photobiol 1987;45:879–889.
2. Dougherty TJ, Kaufman JE, Goldfarb A, Weishaupt KR, Boyle DG, Miffelman A. Photoradiation therapy. a new method for the treatment of malignant tumors. Cancer Res 1978;38:2628–2635.
3. Hayata Y, Kato H, Konaka C, Ono J, Takizawa N. Hematoporphyrin derivative and laser photoradiation in the treatment of lung cancer. Chest 1982;81:269–277.
4. Alian W, Andersson-Engles S, Svanberg K, Svanberg S. Laser-induced fluorescence studies of meso-tetra (hydroxyphenyl) chlorin in malignant and normal tissues in rats. Br J Cancer 1994;70:880–885.
5. Frisoli JK, Tudor EG, Flotte TJ, Hasan T, Deutsch TF, Schomacker KT. Pharmacokinetics of a fluorescent drug using laser-induced fluorescence. Cancer Res 1993;53: 5954–5961.
6. Van der Veen N, van Leengoed HL, Star WM. In vivo fluorescence kinetics and photodynamic therapy using 5-aminolaevnlinic acid-induced porphyrin: increased damage after multiple irradiation. Br J Cancer 1994;70: 867–872.
7. Richards-Kortum R, Mitchell MF, Ramanujam N, Mahadevan A, Thomsen S. In vivo fluorescence spectroscopy: potential non-invasive, automated diagnosis of cervical intraepithelial neoplasia and use as a surrogate endpoint biomarker. J Cell Biochem Suppl 1994;19:111–119.
8. Hayashi J, Kuroiwa Y, Sato H, Saito T, Aizawa K. Transadventitial localization of atheromatous plaques by fluorescence emission spectrum analysis of mono-L-aspartyl chlorin e6. Cardiovasc Res 1993;27:1943–1947.
9. Doiron DR, Profio E, Vincent RG, Dougherty TJ. Fluorescence bronchoscopy for detection of lung cancer. Chest 1979;76:27–32.
10. Kato H, Cortese DA. Early detection of lung cancer by means of hematoporphyrin derivative fluorescence and laser photoradiation. Clin Chest Med 1985;6:237–253.
11. Monnier P, Savary M, Fontolliet C, Wagnieres G, Chatelain A, Cornaz P, Depeursinge C, van den Berg H. Photodetection and photodynamic therapy of "early" squamous cell carcinomas of the pharynx, esophagus and tracheo-bronchial tree. Lasers Med Sci 1990;5:149–168.
12. Kato H, Imaizumi T, Aizawa K, Iwabuchi H, Yamamoto H, Ikeda N, Tsuchida T, Tamachi Y, Ito T, Hayata Y. Photodynamic diagnosis in respiratory tract malignancy using an excimer dye laser system. J Photochem Photobiol B Biol 1990;6:189–196.
13. Bjorkman DJ, Samowitz WS, Brigham EJ, Peterson BJ, Straight RC. Fluorescence localization of early colonic cancer in the rat by hematoporphyrin derivative. Laser Surg Med 1991;11:263–270.
14. Tajiri H, Yokoyama K, Boku N, Ohtsu A, Fujii T, Yoshida S, Sato T, Hakamata K, Hayashi K, Sakata I. Fluorescent diagnosis of experimental gastric cancer using a tumor-localizing photosensitizer. Cancer Lett 1997;111:215–220.
15. Suga S, Xiahedin I, Hayashi N, Kato H, Aizawa K. Effects of mono-L-aspartyl chlorin e6 and laser irradiation on erythrocytes. J Tokyo Med Coll 1996;54:3–8.
16. Hayashi J, Saito T, Kaneda A, Aizawa K. Photodynamic diagnosis and treatment of atheroma. J Jpn Laser Med 1997;18:301–305.
17. Aizawa K, Okunaka T, Ohtani T, Kanawabe H, Yasunaka Y, O'Hata S, Ohtomo N, Nishimiya K, Konaka C, Kato H, Hayata Y, Saito T. Localization of mono-L-aspartyl chlorin e6 (NPe6) in mouse tissues. Photochem Photobiol 1987;46:789–793.
18. Kessel D. Determinants of photosensitization by purpurins. Photochem Photobiol 1989;50:169–174.
19. Nuutinen PJO, Chatlani PT, Bedwell J, MacRobert AJ, Phillips D, Bown SG. Distribution and photodynamic effect of disulphonated aluminum phthalocyanine in the pancreas and adjacent tissues in the Syrian golden hamster. Br J Cancer 1991;64:1108–1115.
20. Sahai D, Lo JL, Hagen IK, Bergstrom L, Chernomorsky S, Porezt RD. Metabolically convertible lipophilic derivatives of pH-sensitive amphipathic photosensitizers. Photochem Photobiol 1993;58:803–808.
21. Gomer CJ, Ferrario A. Tissue distribution and photosensitizing properties of mono-L-aspartyl chlorin e6 in a mouse tumor model. Cancer Res 1990;50:3985–3990.
22. Katsumi T, Aizawa K, Kuroiwa Y, Saito K, Kurata Y, Li Y, Okunaka T, Konaka C, Kato H. Photodynamic therapy with a diode laser for implanted fibrosarcoma in mice employing mono-L-aspartyl chlorin e6. Photochem Photobiol 1996;64:671–675.
23. Spikes JD, Bommer JC. Photobleaching of mono-L-aspartyl chlorin e6 (NPe6) A candidate sensitizer for the photodynamic therapy of tumors. Photochem Photobiol 1993;58:346–350.
24. Roberts WG, Shiau F-Y, Nelson JS, Smith KM, Berns MW. In vitro characterization of monoaspartyl chlorin e6 and diaspartyl chlorin e6 for photodynamic therapy. JNCI 1988;80:330–336.
25. Furukawa K, Okunaka T, Shibuya H, Matsuzaka E, Ikeda N, Konaka C, Kato H. Experimental photodynamic diagnosis of malignant tumor using new photosensitizer. J Jpn Laser Med 1996;17:59–63.
26. Rous P, Beard JW. The progression to carcinoma of virus induced rabbit papillomas (Shope). J Exp Med 1935;62: 523–548.
27. Sugawara K. Integration and methylation of Shope pap-

illoma virus DNA in the transplantable VX2 and VX7 rabbit carcinomas. Virology 1983;131:88–99.
28. Lam S, MacAulay C, Hung J, Leriche J, Profio AE, Palcic B. Detection of dysplasia and carcinoma in situ with a lung imaging fluorescence endoscope device. J Thorac Cardiovasc Surg 1993;105:1035–1040.
29. Schomacker KT, Frisoli JK, Compton CC, Flotte TJ, Richter JM, Nishioka NS, Deutsh TF. Ultraviolet laser-induced fluorescence of colonic tissue: basic biology and diagnostic potential. Lasers Surg Med 1992;12:63–78.
30. Nelson JS, Roberts WG, Berns MW. In vivo studies on the utilization of mono-L-aspartyl chlorin e6 (NPe6) for photodynamic therapy. Cancer Res 1987;47:4681–4685.
31. Aizawa K. Spectral analysis of constituent organic molecule with an electromagnetic wave. J Physiol Soc Jpn 1994;56:49–63.